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Mechanisms of acquired resistance to EGFR tyrosine kinase inhibitors in EGFR-mutant non-small cell lung cancer

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MECHANISMS OF ACQUIRED RESISTANCE TO EGFR TYROSINE KINASE INHIBITORS IN EGFR-MUTANT NON-SMALL CELL LUNG CANCER

by

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MECHANISMS OF ACQUIRED RESISTANCE TO EGFR TYROSINE KINASE INHIBITORS IN EGFR-MUTANT NON-SMALL CELL LUNG CANCER

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ABSTRACT

Non-small cell lung cancer (NSCLC) makes up the majority of lung cancers, which remains the leading cause of cancer mortality worldwide. NSCLC with mutant-epidermal growth factor receptor (EGFR) is currently treated with tyrosine kinase inhibitors (TKIs). TKIs have proven effective in improving survival until resistance is conferred, mostly by way of the exon 20, threonine 790 to methionine (T790M) point mutation in EGFR. The mechanism by which this point mutation arises is poorly understood. Herein we report a possible pathway by which the C to T transition that leads to T790M comes about. We show that activation-induced cytidine deaminase (AID) mRNA expression is induced upon treatment with EGFR TKIs in mutant-EGFR human lung cancer cell lines but not in control cell lines. We also show that stable expression of AID is sufficient to produce resistance to one such TKI, erlotinib, and is sufficient to produce T790M itself. We also report that B-cell lymphoma 6 (BCL6) may precede AID in this pathway. Our results show that BCL6 is upregulated in these cell lines treated with EGFR TKIs but not in normal bronchial cells. We then treated human lung cancer cell lines with EGFR TKIs in combination with BCL6 inhibitors. Our results show that AID is dependent upon BCL6 expression. Finally, we report on results from a transient BCL6 overexpression which lead us to believe that AID mRNA receives input from at least one alternate pathway in addition to BCL6. We also performed these experiments on a family

of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) cytidine deaminases, that show they may be involved in this pathway downstream of AID. Taken together, our results suggest a potential pathway involving BCL6, AID, and APOBEC cytidine deaminases that lead to the C to T transition that produces T790M, thereby conferring resistance to EGFR TKIs in mutant-EGFR NSCLC. They also provide potential new targets for treatment should further study confirm our results.

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LIST OF ABBREVIATIONS

AICDA..... Activation-induced cytidine deaminase (gene name for AID)

AID ..... Activation-induced cytidine deaminase (protein)

APOBEC..... Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

ATP ..... Adenosine triphosphate

BCL6..... B-cell lymphoma 6

cDNA ..... Complementary deoxyribonucleic acid

Del19..... EGFR exon 19 deletion

EGFR ..... Epidermal growth factor receptor

GAPDH..... Glyceraldehyde 3-phosphate dehydrogenase

GC ..... Germinal center

L858R ..... Leucine 858 to arginine point mutation

mAID ..... Murine activation-induced cytidine deaminase

mRNA ..... Messenger ribonucleic acid

NFκB..... Nuclear factor kappa-light chain enhancer of activated B-cells

NSCLC..... Non-small cell lung cancer

PC9-ER ..... PC9 erlotinib-resistant

PIK3CA..... Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

RNA ..... Ribonucleic acid

RPMI..... Roswell Park Memorial Institute

SCLC..... Small-cell lung cancer

T790M..... Threonine 790 to methionine point mutation

TKI..... Tyrosine kinase inhibitor

TP53..... Tumor protein 53

INTRODUCTION

The leading cause of cancer mortality worldwide for both men and women remains lung cancer, the vast majority of which is diagnosed as non-small cell lung cancer (NSCLC). At late stages, the 5-year survival for NSCLC is 10% or less (American Cancer Society, 2018). The discovery of a subset of NSCLC with an epidermal growth factor receptor (EGFR) gene somatic activating mutation was a significant breakthrough towards more targeted treatment for such patients. About 10-15% of Caucasians and 30-50% of Asians with NSCLC have this mutation, most commonly affecting gene exons 18-21 on chromosome 7, encoding the adenosine triphosphate (ATP) binding domain on the tyrosine kinase receptor (Sequist et. al., 2007). The EGFR exon 19 deletion (Del19) and exon 21 leucine 858 to arginine point mutation (L858R) together account for 90% of all EGFR mutations in NSCLC (Zhuo et. al., 2017).

Current first-line therapy for EGFR-mutant NSCLC includes approved tyrosine kinase inhibitors (TKIs) erlotinib, gefitinib, and afatinib. Despite the improvement in survival these drugs have provided, prognosis remains poor with median progression-free survival of 10-13 months (Maemondo et. al., 2010; Rosell et. al., 2012; Sequist et. al., 2013). Resistance to these drugs occurs primarily by an exon 20 Thr790Met point mutation (T790M). This mutation is responsible for about 50-60% of cases of drug resistance in this setting (Figure 1).

Other mutations observed upon resistance to treatment with TKIs include *MET* (hepatocyte growth factor receptor) amplification, transformation to small-cell lung cancer (SCLC), PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) mutations, and a combination of these (Sequist et. al., 2011).

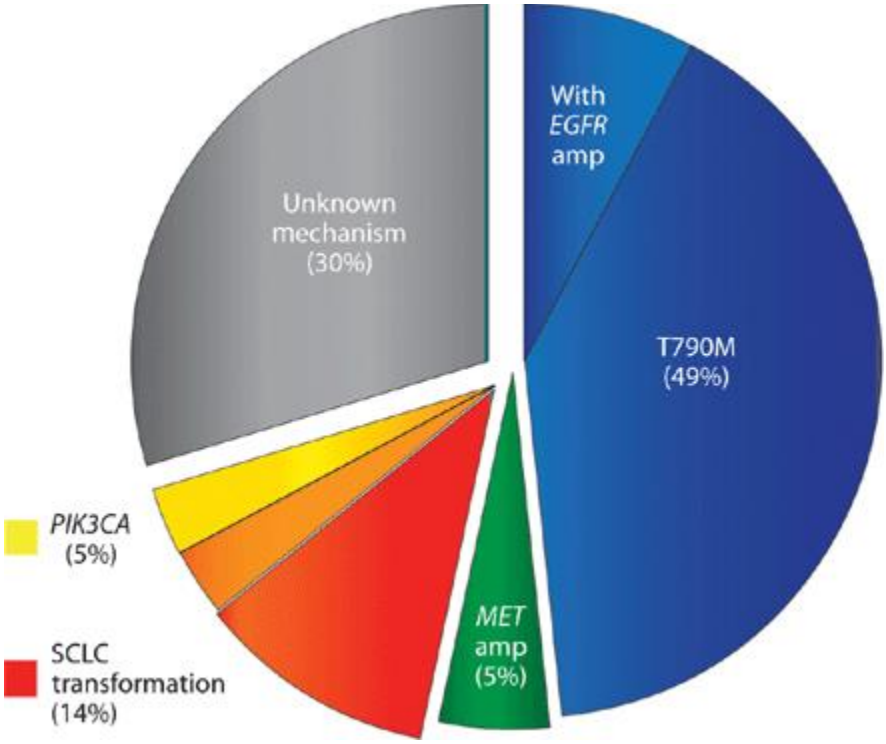


Figure 1: Frequency of mutations observed upon acquisition of resistance to TKIs in EGFR-mutant NSCLC patients. Biopsy samples were taken from 37 patients at the time of resistance and compared with samples taken prior to treatment. The majority were found with T790M, while 14% were found with SCLC transformation, 5% with *MET* amplification, and 5% with PIK3CA. The orange slice represents a single patient found with both SCLC transformation and a PIK3CA mutation (Sequist et. al., 2011).

Although third and fourth-line TKI drugs are emerging, there is a clear need for better understanding of the mechanisms of acquired resistance in hopes

of new and improved targeted therapy. This thesis will attempt to elucidate mechanisms by which exon 20 T790M occur in TKI-resistant NSCLC. One possibility to be explored will involve the role that activation-induced cytidine deaminase (AID) may play in the acquisition of this mutation. The gene name for AID is *AICDA*, and as such, AID mRNA expression will be referred to as AICDA in this thesis. AID induces mutations required for the generation of antibody diversity in B lymphocytes. It does so by deaminating cytosine to produce uracil, resulting in C/G to T(U)/A transitions in DNA and/or RNA. As such, it introduces genomic instability that can result in chromosomal translocations and other mutations involving proto-oncogenes. While AID is normally tightly regulated, aberrant expression has been found in human lung cancers. In addition, AID protein expression levels were found to be associated with tumor protein 53 (TP53) mutations in primary lung cancers (Shinmura et. al., 2011). This thesis will explore if AID may contribute to the development of mutations such as T790M, and by which mechanisms it may do so.

One such mechanism could involve BCL6, which has emerged as a key oncoprotein due to its broad and powerful role as a transcriptional repressor for hundreds of genes, including those that target DNA damage sensing and proliferation checkpoints. BCL6, named for its initial identification in diffuse large B-cell lymphomas, normally functions in the humoral immune response. BCL6 upregulation is required for the formation of germinal centers (GC) upon exposure to antigens. It also regulates normal proliferation and mutagenesis,

carried out by AID under its direction, of B cells during the humoral immune response (Cardenas et. al., 2016). The important connection between BCL6 and AID leads to the question of whether aberrant expression of BCL6 may lead to a subsequent change in expression or behavior of AID in solid tumors, particularly NSCLC.

Related to AID, and potentially also contributing to a pathway leading to T790M, is a family of APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) cytidine deaminases which also convert cytosine bases to uracil and normally function in innate immunity and RNA editing. Within a subclass of this family of proteins are several homologs with specificity for a mutated cytosine directly following a thymine (tC), including APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, and APOBEC3H. However, APOBEC3G and AID do not share this specificity (Roberts et. al., 2013). In 2013, Roberts *et. al.* reported on distinctive patterns of APOBEC mutagenesis in many cancer genomes, and that these patterns correlate with an increase in APOBEC mRNA expression and include cancer driver genes. We suggest that, in addition to and possibly as a result of BCL6 upregulation, both AID and this family of APOBEC proteins contribute to the mutagenesis that gives rise to T790M which confers resistance to EGFR TKIs in mutant-EGFR NSCLC. By contributing to the body of knowledge on NSCLC drug resistance, there will be more opportunity for discovery of potential therapeutic targets.

METHODS

Cell Culture

PC9, H3255, HCC827, H1975, H460, BaF3, and BEAS2B cells were grown at 37 ºC in a humidified incubator at 5% CO_2 in Roswell Park Memorial Institute (RPMI) media (Corning) supplemented with 10% fetal bovine serum (Corning), 100 µg/ml penicillin and 100 units/ml streptomycin (Corning).

Quantitative PCR

Total RNA was trypsinized and isolated from cells, grown on agar plates for 24 hours, using an RNA isolation kit (Qiagen, RNeasy Mini Kit), up to 4.5 µg of which was reverse transcribed to cDNA following the Invitrogen Superscript™ II Reverse Transcriptase protocol. RNA input across samples was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels. iTaq™ Universal SYBR® Green Supermix (BIO-RAD) was used for gene expression analysis across all genes, and mRNA expression levels were quantified, using a quantitative PCR thermal cycler, in triplicates with 150 ng cDNA in a 15 µl reaction volume. Primers are shown in Table 1. Statistical significance was found using the Student's *t*-test. A cell-permeable, selective inhibitor of the transcriptional repression activity of BCL6, 'BCL6 inhibitor 79-6,' was purchased from Calbiochem.

Gene	Primers	5’-3’
GAPDH	**F**	**CCAGGCGCCCAATACG**
	R	**CCACATCGCTCAGACACCAT**
BCL6	**F**	**TTCCGCTACAAGGGCAAC**
	R	**TGCAACGATAGGGTTTCTCA**
AICDA	**F**	**AAATGTCCGCTGGGCTAAGG**
	R	**GGAGGAAGAGCAATTCCACGT**
APOBEC3A	**F**	**GAGAAGGGACAAGCACATGG**
	R	**TGGATCCATCAAGTGTCTGG**
APOBEC3B	**F**	**GACCCTTTGGTCCTTCGAC**
	R	**GCACAGCCCCAGGAGAAG**
APOBEC3C	**F**	**AGCGCTTCAGAAAAGAGTGG**
	R	**AAGTTTCGTTCCGATCGTTG**
APOBEC3D	**F**	**ACCCAAACGTCAGTCGAATC**
	R	**CACATTTCTGCGTGGTTCTC**
APOBEC3F	**F**	**CCGTTTGGACGCAAAGAT**

	R	**CCAGGTGATCTGGAAACACTT**
APOBEC3G	**F**	**CCGAGGACCCGAAGGTTAC**
	R	**TCCAACAGTGCTGAAATTCG**

Table 1: Primers used in quantitative PCR analysis for genes of interest

RESULTS

Erlotinib induces up-regulation of AID mRNA (AICDA) in human lung cancer cell lines harboring EGFR mutations.

It has been shown that AID mRNA, which I will refer to by AID's gene name AICDA, is detected in lung cancer cell lines and primary lung cancer specimens (Kou et. al., 2007). This raises the question of whether it could be responsible for EGFR mutagenesis. Our group first examined AICDA expression in lung cancer cell lines harboring activating EGFR mutations (Table 2).

Table 2: Human lung cancer cell lines that are used in this thesis.

Cells	BEAS-2B	H460	HCC827	PC9	PC9-ER	H3255	H1975	Ba/F3-L858R
EGFR	Wild-type	Wild-type	Del19	Del19	Del19/ L858R	L858R	L858R/ T790M	L858R
Other mutations	None	Kras	None	None	None	None	None	None

We found AICDA expression present but at low levels in most cell lines (Figure 2: blue bars). When these cells were treated with erlotinib, AICDA expression increased 5 to 25-fold in cells harboring EGFR mutations (PC9, H2355, HCC827, H1975). There was no statistically significant change in H460 cells harboring wild-type EGFR (Figure 2: red bars).

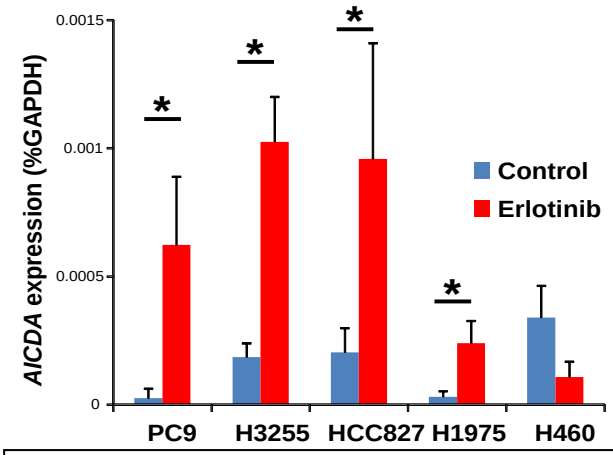


Fig.2 Expression of AICDA in human lung cancer cell lines. Cells were treated with 1 μM erlotinib (0.1 μM for H3255) for 24 hours and expression of AICDA was evaluated by real time PCR analysis. *p<0.05 vs. control.

Next, our group studied whether AICDA is induced by treatment with EGFR TKIs, and whether AICDA then triggers the emergence of the T790M acquired resistance mutation. To do so, our group generated PC9 cells resistant to erlotinib (PC9-ER) by starting with parental PC9 cells, which harbor an exon 19 deletion and are sensitive to erlotinib. They were treated initially with the inhibitory concentration 30% (IC_{30}) of 0.01μM

erlotinib. Upon resuming close to normal growth kinetics over a period of about 2 weeks per concentration, the treatment was escalated stepwise to concentrations of 0.03μM, 0.1μM, 0.3μM, 1μM, and finally 3μM over a total period of 3 months. PC9-ER cells were then grown in 1μM erlotinib at nearly normal growth kinetics (Figure 3A). Then, our group sequenced DNA and RNA from parental PC9 and PC9-ER cells to reveal the introduction of the T790M mutation as caused by a characteristic C to T base-pair change. Of note, only a minority of the cell population acquired this change. This is because EGFR is heterozygous, and many cells do not survive treatment or retain a wild-type allele and thus do not become fully resistant (Figure 3B). Also, in cells resistant to erlotinib, AICDA expression increased over 60-fold compared to that in parental cells

(Figure 3C). Altogether, these findings suggest that AID could be involved in the acquisition of T790M and resistance to EGFR TKIs.

Expression of AID induces EGFR-T790M mutation in Ba/F3-L858R cells.

Next, our group sought to find out whether AID is sufficiently capable of generating EGFR-T790M by using Ba/F3 cells expressing EGFR-L858R (Ba/F3-LR). Ba/F3 cells are murine pro-B cells that become solely

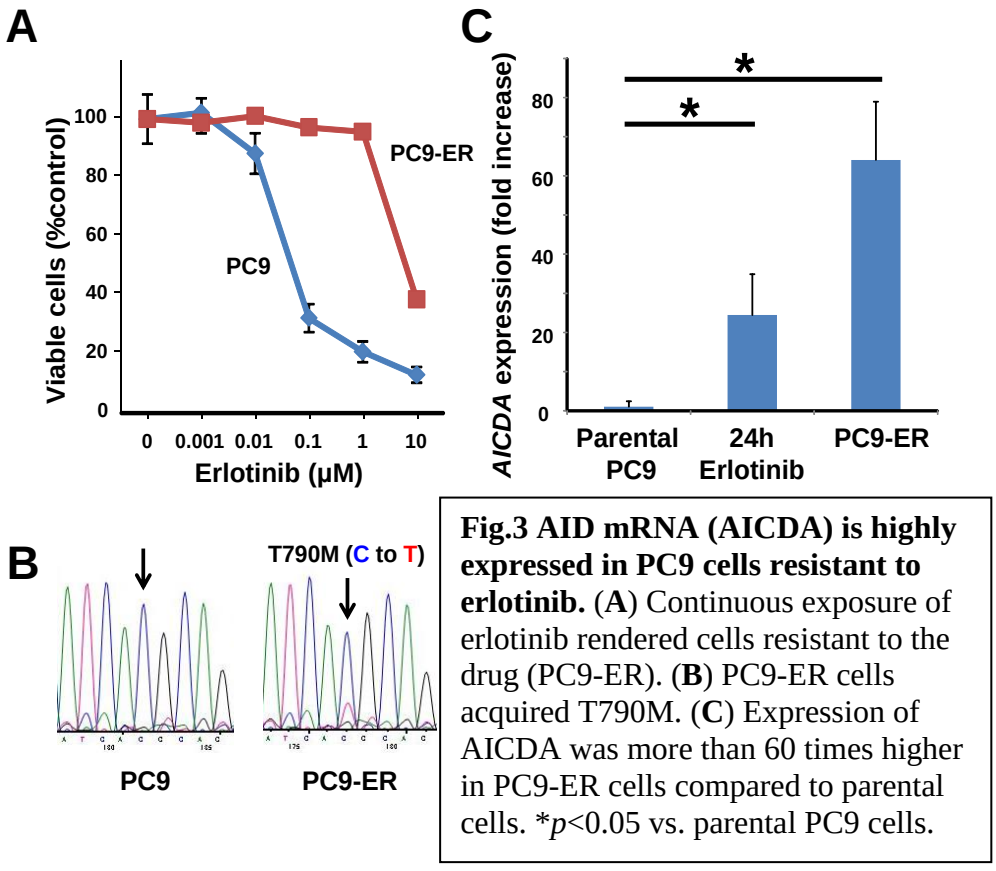


Fig.3 AID mRNA (AICDA) is highly expressed in PC9 cells resistant to erlotinib. (**A**) Continuous exposure of erlotinib rendered cells resistant to the drug (PC9-ER). (**B**) PC9-ER cells acquired T790M. (**C**) Expression of AICDA was more than 60 times higher in PC9-ER cells compared to parental cells. *p<0.05 vs. parental PC9 cells.

dependent on introduced constitutively activated kinases for survival in place of their normal dependence on interleukin 3 (IL-3). In this case, our

group sought to replicate the common mutation in TKI-naïve mutant-EGFR NSCLC, EGFR-L858R, in Ba/F3 cells to isolate the impact of EGFR mutations on their function and downstream signaling pathways. Another advantage to using Ba/F3 cells is that they do not express endogenous AID, so our group decided to stably express AID DNA in one group of cells to discover whether AID can generate T790M compared to otherwise identical cells that do not express AID. Thus, either murine AID (Ba/F3-LR-mAID) or a control (Ba/F3-LR-control) were introduced via lentiviral infection. Upon treatment with erlotinib, both cell lines were initially sensitive and underwent apoptosis, but Ba/F3-LR-mAID cells became erlotinib-resistant after only 3 weeks (Figure 4). Resistant cells were subcloned and mRNA was isolated from individual clones for sequencing of their cDNA, which revealed that 100% of clones (8/8) harbored an EFGR C to T substitution that led to T790M emergence (Figure 5). These results indicate that AID causes the C to T mutation that leads to T790M.

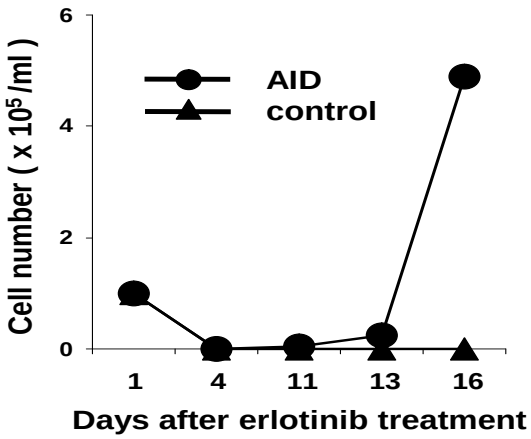


Fig.4 Expression of AID in Ba/F3-L858R cells confers resistance to erlotinib. Ba/F3-L858R cells expressing mAID or control cells (1 x 10^5/ml) were treated with 1 μM erlotinib and viable cells were counted by trypan blue exclusion.

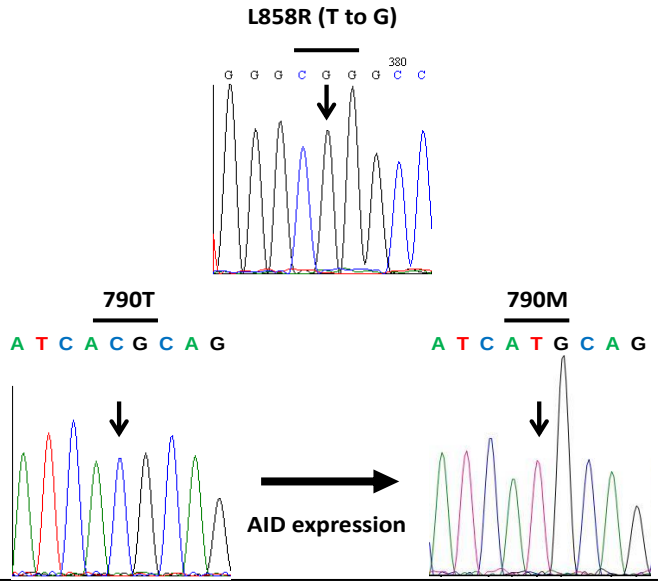


Fig.5 Expression of AID in Ba/F3-L858R cells generated T790M mutation. The top figure shows the sequence of one clone from the Ba/F3-LR-control cells. Ba/F3-LR-mAID cells were treated with 1 μM erlotinib and resistant cells were subcloned. Sequences of cDNA revealed that 100% of the clones (8/8) harbor a C to T substitution leading to T790M. Shown is the sequence of one clone from these cells before and after resistance.

EGFR TKIs induce up-regulation of BCL6 mRNA in human lung cancer cell lines harboring EGFR mutations.

The next question we sought to answer was whether AID might be part of a signaling mechanism that involves other important molecules of interest. B-cell lymphoma 6 (BCL6), a powerful transcriptional repressor with broad effects, has emerged as a possible therapeutic target for a growing list of tumor types, among them non-small cell lung cancer. Specifically, it was shown to be required for survival and proliferation of NSCLC cells, due to its role in repressing DNA damage response genes. Its normal function in the humoral immune response involves control over AID's DNA-editing role that allows B cells to undergo immunoglobulin affinity maturation (Cardenas et. al., 2016). For these reasons, we sought to understand whether BCL6 may be a driver of AID action to bring about the acquired T790M mutation after exposure to EGFR TKIs. The first way we investigated this was to determine whether BCL6 mRNA levels were increased, as AICDA levels were, upon treatment with EGFR TKIs in human lung cancer cell lines harboring EGFR mutations. To test this, we treated PC9 and HCC827 cells with erlotinib and afatinib, H1975 cells with osimertinib, and BEAS-2B cells, which are normal bronchial epithelial cells, with erlotinib (Figure 6). Each drug was given at increasing concentrations for 24 hours. We found an increase in BCL6 mRNA expression upon treatment with EGFR TKI from about 2 to over

10-fold across human lung cancer cell lines harboring EGFR mutations, while the normal bronchial epithelial cell line showed about a 1.25-fold increase in BCL6 expression at its peak but was reduced again at the highest concentration of drug given. Our findings show that EGFR TKI treatment consistently induces an up-regulation in BCL6 mRNA in EGFR-mutant human lung cancer cell lines.

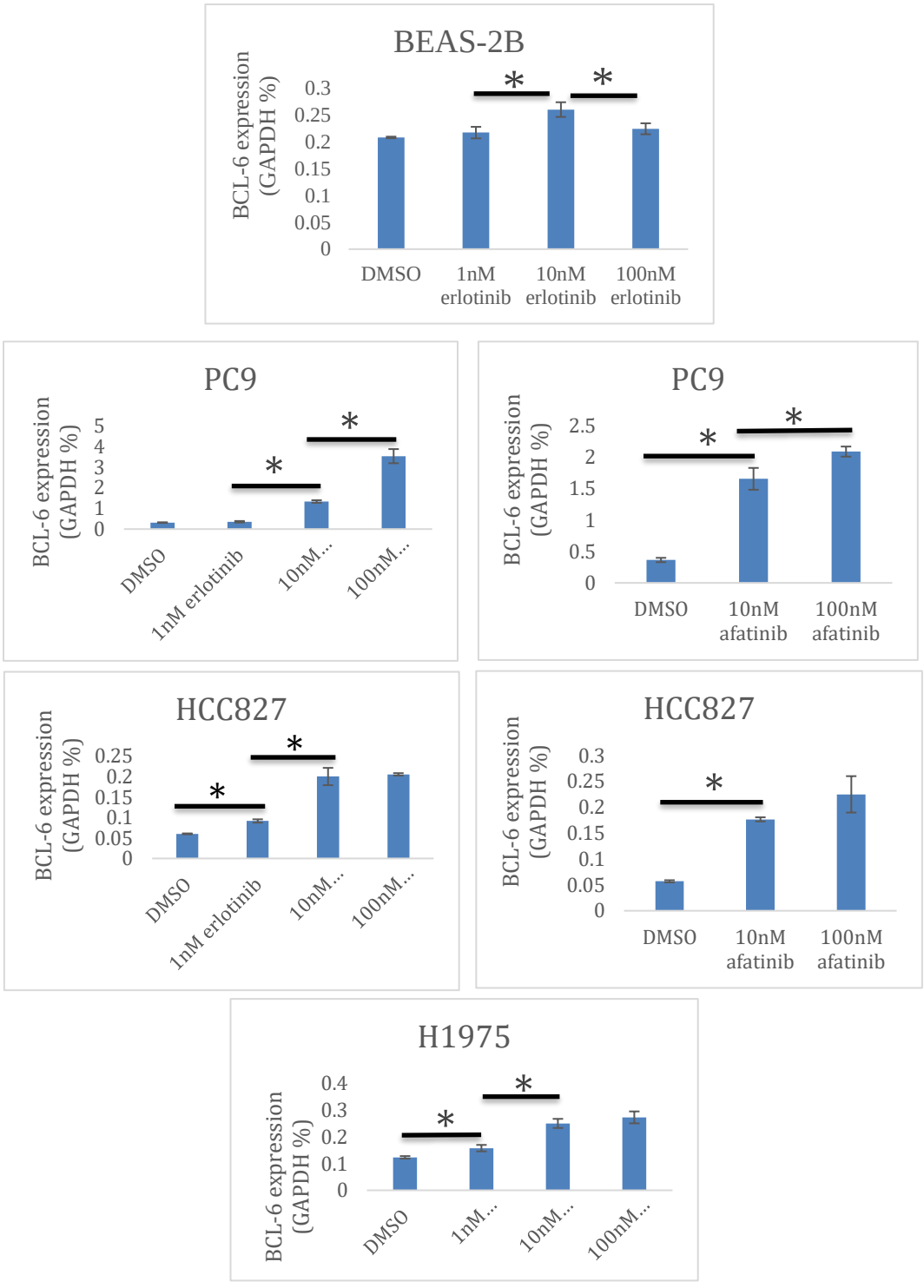


Figure 6: Expression of BCL6 is upregulated across EGFR-mutant human lung cancer cell lines when treated with EGFR TKIs at

increasing concentrations for 24 hours. Normal bronchial epithelial cells, BEAS-2B, do not exhibit an upregulation of BCL6 of similar magnitude or persistence upon treatment with erlotinib. *$p<0.05$ vs. control.

EGFR TKIs increase AICDA expression in largely dose-independent fashion across multiple human lung cancer cell lines harboring EGFR mutations.

We next sought to investigate the dose-responsiveness of AICDA upregulation in the same treatment conditions as those in which BCL6 was upregulated. We again treated human lung cancer cell lines harboring EGFR mutations for 24 hours with increasing doses of EGFR TKIs, then measured AICDA expression. As previously shown, AICDA levels rose up to 6-fold in some cell lines, although others showed lesser or no significant increases (Figure 7). This variability is to be expected owing to characteristic low endogenous levels of AICDA, as low as 0.0001% of GAPDH (Figure 7). While these results echo those previously shown in Figure 2, the variability in dose-response and between cell lines may allude to a more complex and less direct relationship between treatment with EGFR TKIs and AICDA expression.

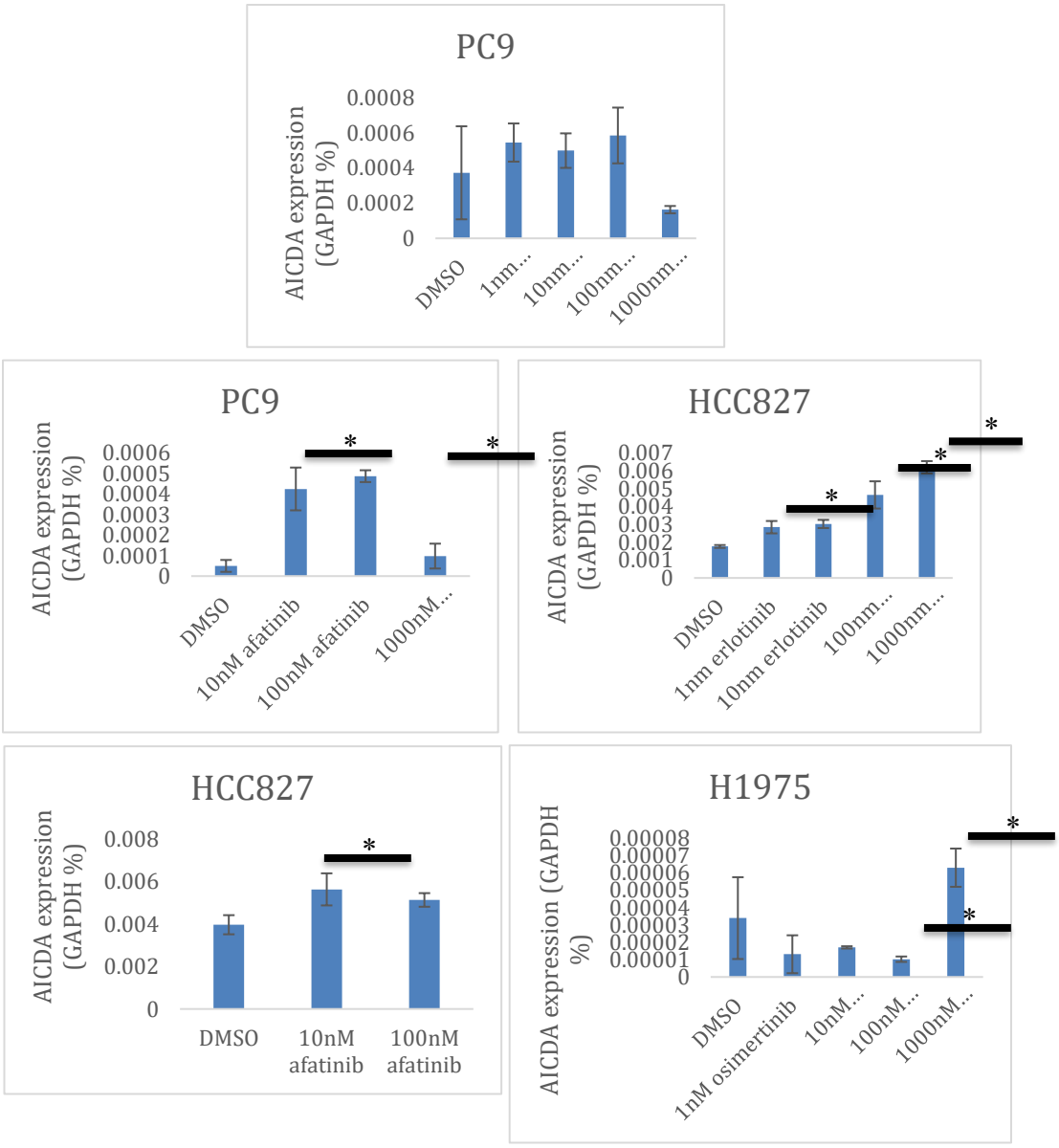


Figure 7: Expression of AICDA is upregulated in most EGFR-mutant human lung cancer cell lines when treated with EGFR TKIs at increasing concentrations for 24 hours. Of note, PC9 cells shown in the top plot were treated with erlotinib. HCC827 cells exhibit the most consistent upregulation in AICDA expression, but PC9, when treated with afatinib, and H1975, when treated with osimertinib, exhibit significant upregulation as well. *p<0.05 vs. preceding dose.

Patterns of APOBEC family mRNA expression upon EGFR TKI treatment in human lung cancer cell lines.

Next, we sought to determine whether AID may act alongside members of the APOBEC family to induce the C to T transition that leads to T790M. To do so, we screened for similar increases in mRNA expression upon EGFR TKI treatment across the APOBEC family of proteins in the same cell lines. Again, we treated human lung cancer cell lines for 24 hours at increasing doses of EGFR TKIs and measured mRNA expression in APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, and APOBEC3G. Despite low expression in some cell lines before treatment, we found promising increases in mRNA expression in APOBEC3A (Figure 8), APOBEC3B (Figure 9), APOBEC3C (Figure 10), APOBEC3D (Figure 11), APOBEC3F (Figure 12), and APOBEC3G (Figure 13). Most notably, APOBEC3B showed up to a 4-fold increase in mRNA expression at the largest dose of EGFR TKI, while APOBEC3D and APOBEC3F showed up to a 3-fold increase in mRNA expression upon treatment at the highest dose. Given this evidence, there is reason to believe that APOBEC3B, APOBEC3D, and APOBEC3F may be important in the development of the T790M mutation after treatment with EGFR TKIs.

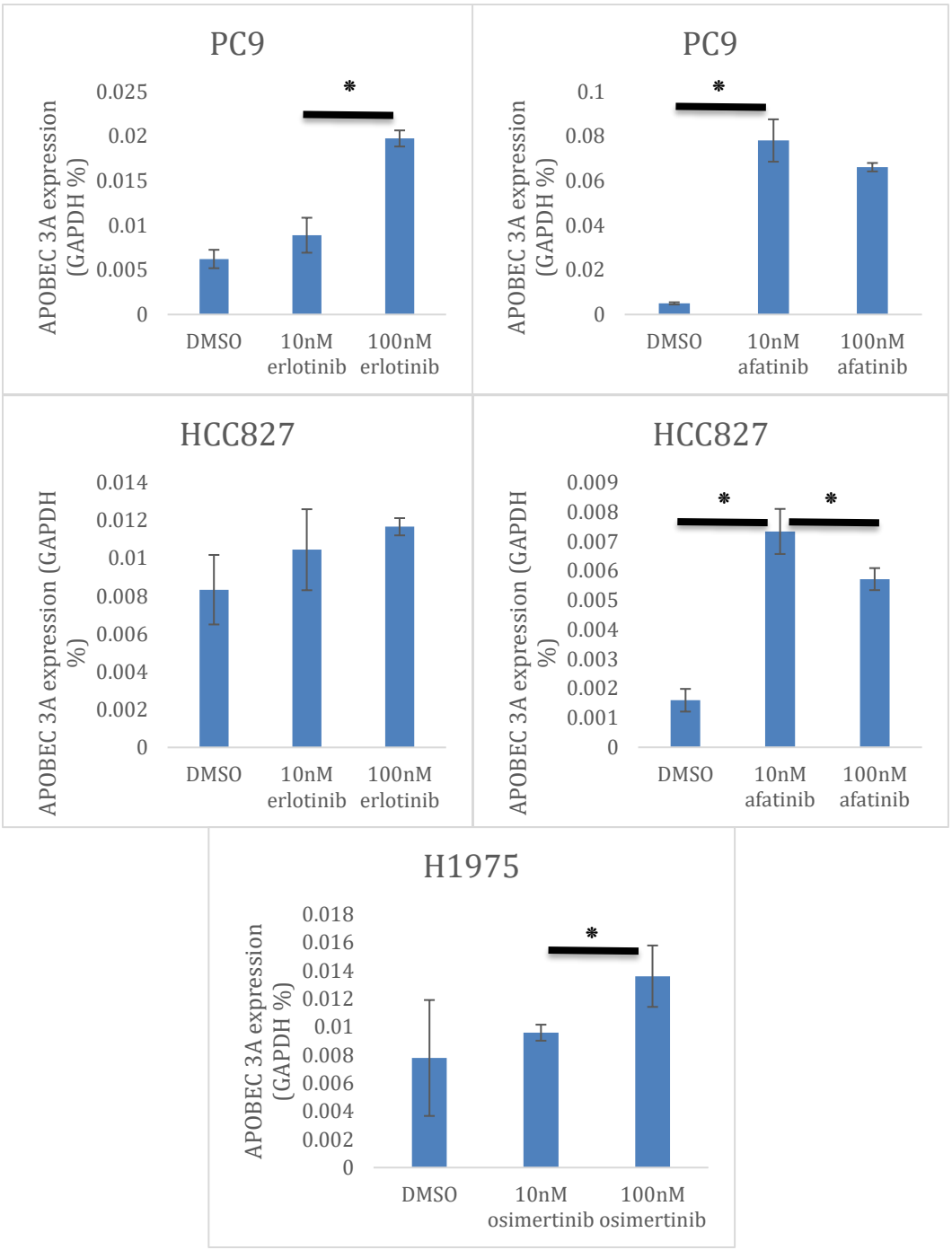


Figure 8: APOBEC3A mRNA expression is upregulated across human lung cancer cell lines upon treatment with EGFR TKI for 24 hours at increasing concentrations. With the exception of HCC827 cells treated with erlotinib, all cell lines tested showed significant increases in mRNA expression at increasing doses of EGFR TKI. *$p<0.05$ vs. preceding dose.

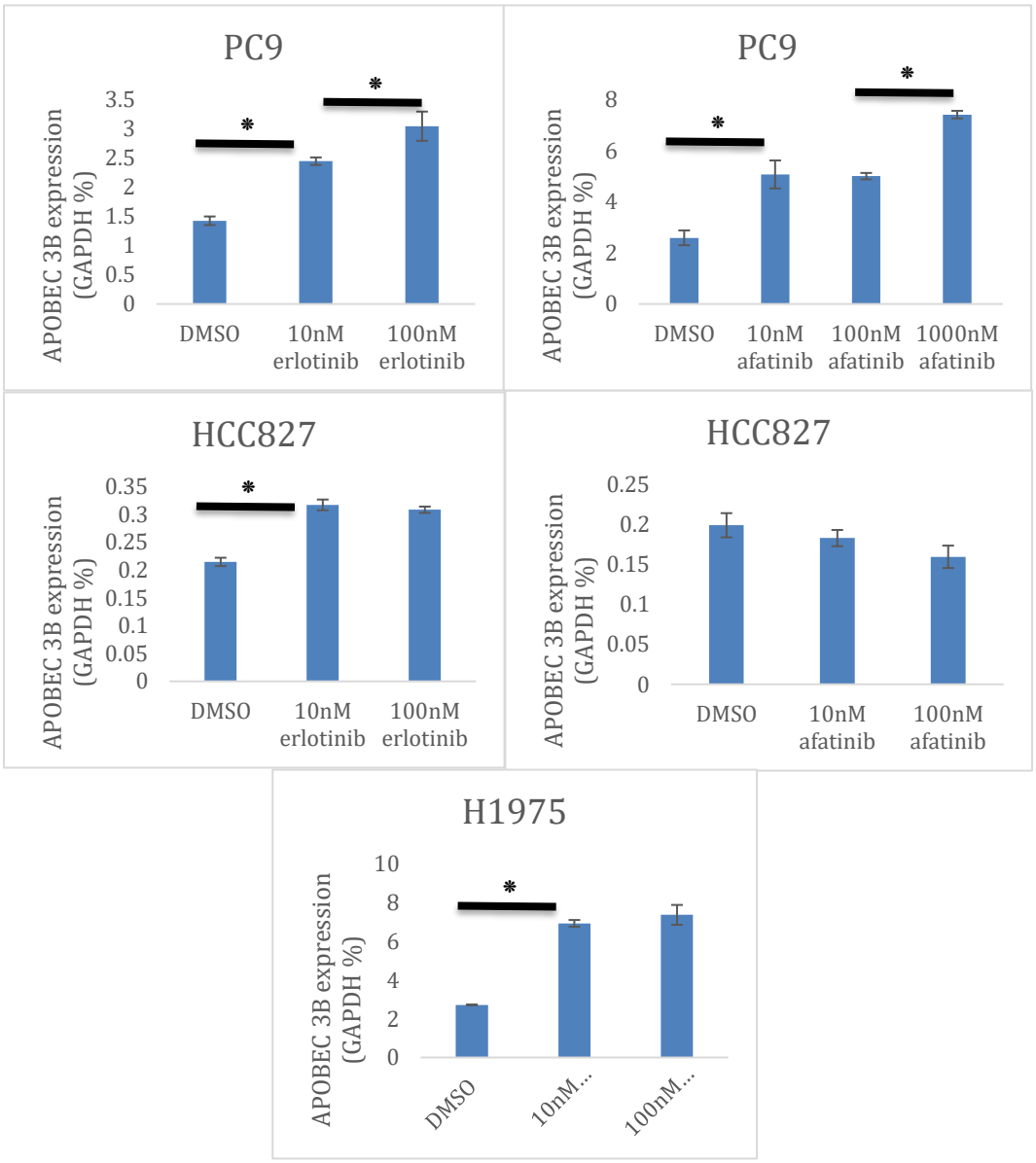


Figure 9: APOBEC3B mRNA expression is upregulated across human lung cancer cell lines upon treatment with EGFR TKI for 24 hours at increasing concentrations. With the exception of HCC827 cells treated with afatinib, all cell lines tested showed significant increases in mRNA expression at increasing doses of EGFR TKI. *p<0.05 vs. preceding dose.

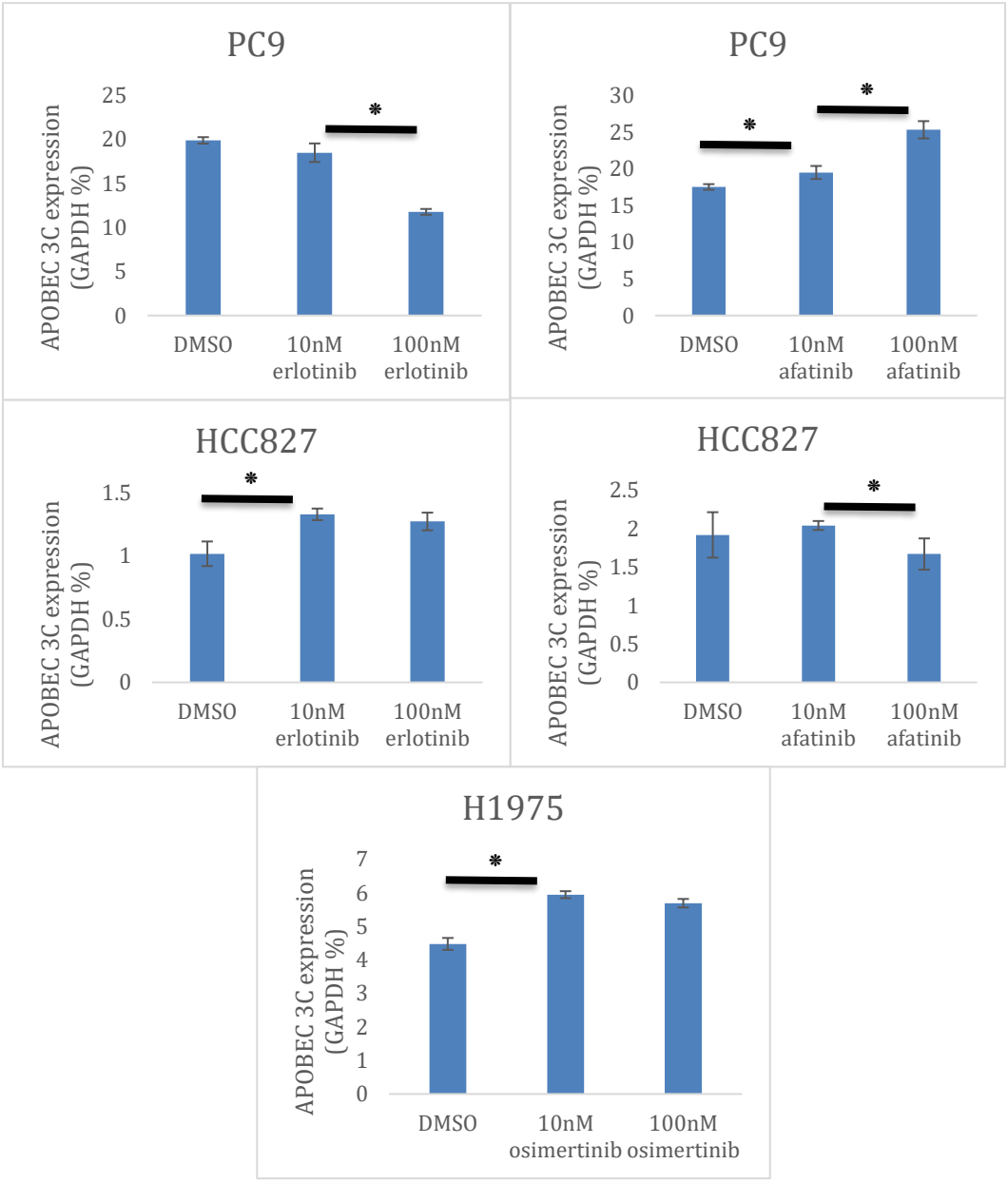


Figure 10: APOBEC3C mRNA expression is upregulated across human lung cancer cell lines upon treatment with EGFR TKI for 24 hours at increasing concentrations. With the exception of HCC827 cells treated with afatinib and PC9 cells treated with erlotinib, all cell lines

tested showed significant increases in mRNA expression at increasing doses of EGFR TKI. *p<0.05 vs. preceding dose.

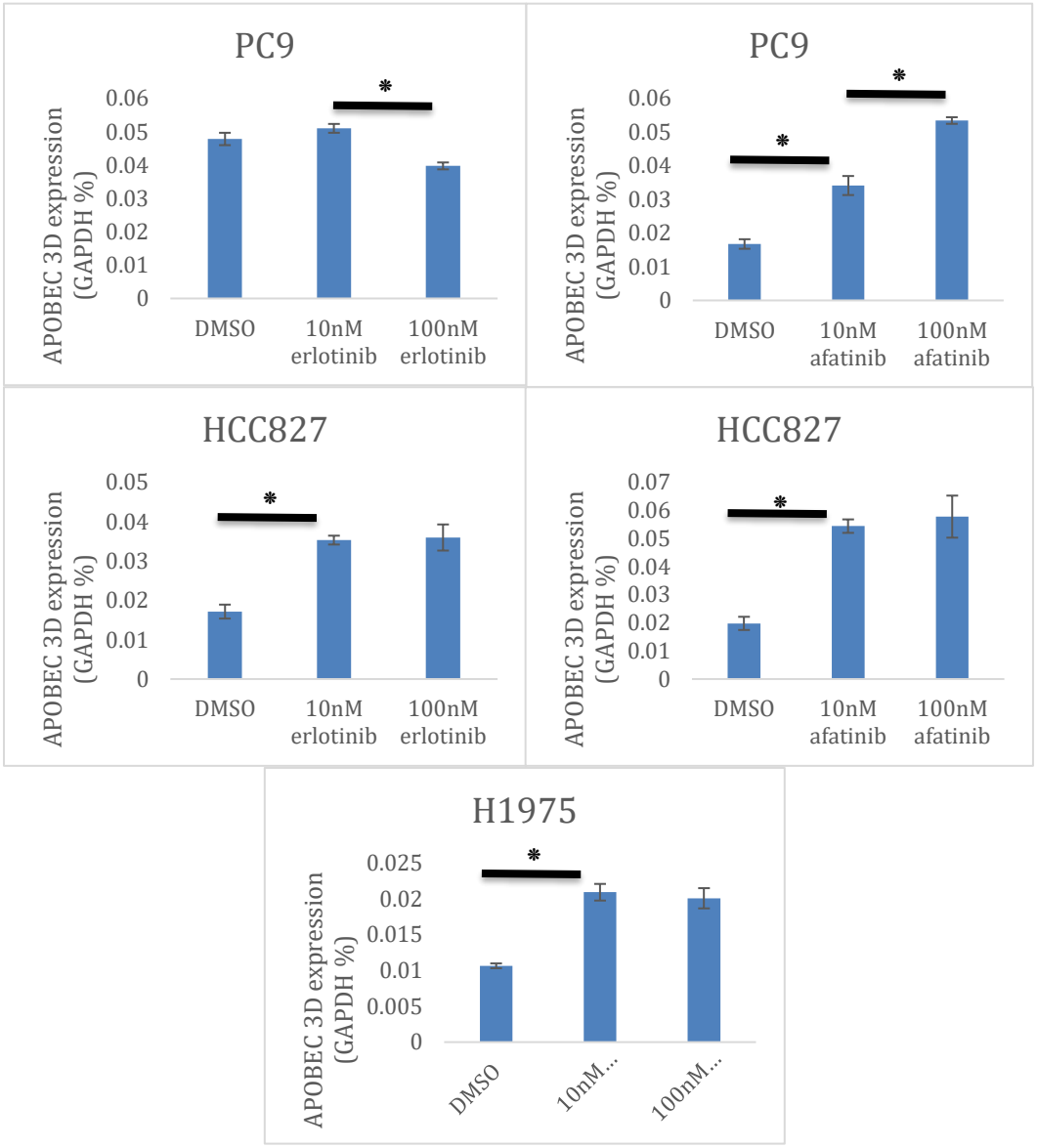


Figure 11: APOBEC3D mRNA expression is upregulated across human lung cancer cell lines upon treatment with EGFR TKI for 24 hours at increasing concentrations. With the exception of PC9 cells treated with erlotinib, all cell lines tested showed significant increases in mRNA expression at increasing doses of EGFR TKI. *p<0.05 vs. preceding dose.

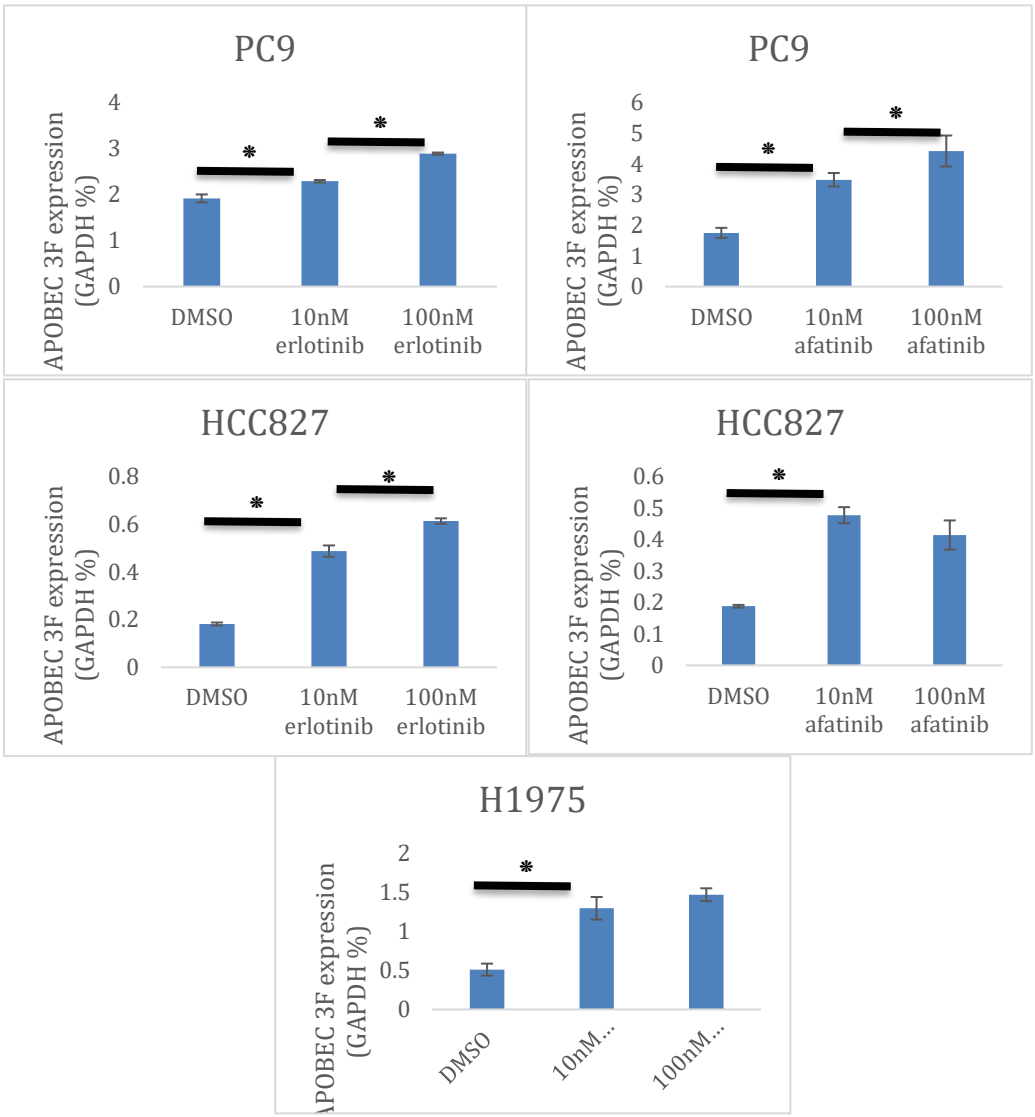


Figure 12: APOBEC3F mRNA expression is upregulated across human lung cancer cell lines upon treatment with EGFR TKI for 24 hours at increasing concentrations. All cell lines tested showed

significant increases in mRNA expression at increasing doses of EGFR TKI. *p<0.05 vs. preceding dose.

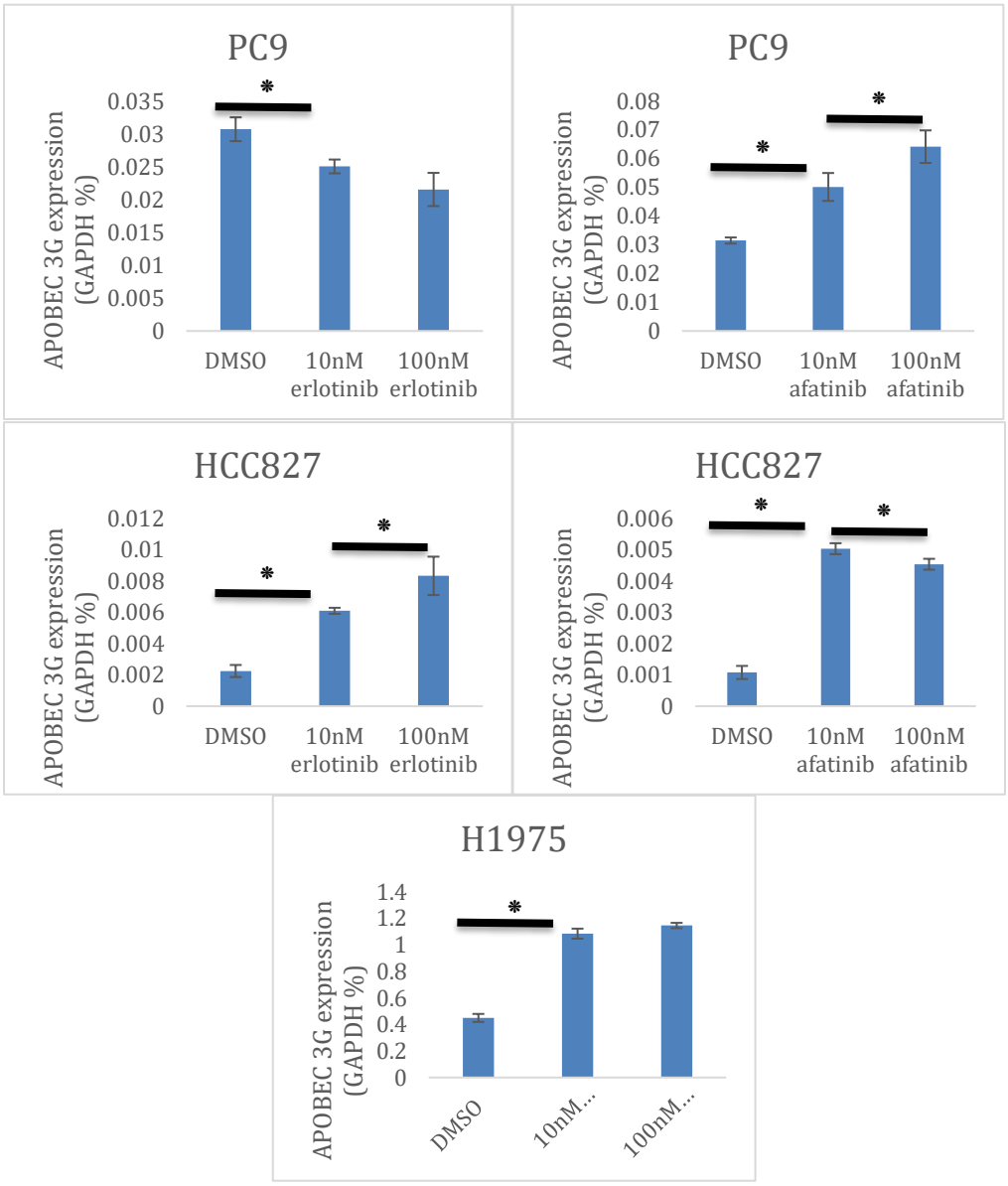
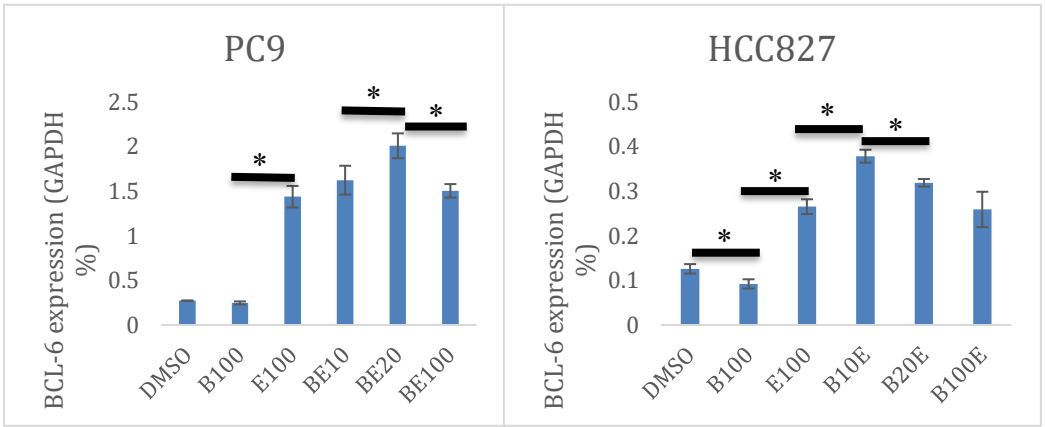


Figure 13: APOBEC3G mRNA expression is upregulated across human lung cancer cell lines upon treatment with EGFR TKI for 24 hours at increasing concentrations. With the exception of PC9 cells

treated with erlotinib, all cell lines tested showed significant increases in mRNA expression at increasing doses of EGFR TKI. *p<0.05 vs. preceding dose.

BCL6 mRNA upregulation persists upon EGFR TKI treatment despite pharmacologic BCL6 inhibition.

Next, we decided to test the extent to which EGFR TKIs induce BCL6 mRNA upregulation by treating lung cancer cell lines for 24 hours with DMSO only, 100µM of a pharmacologic inhibitor of BCL6 (B100), 100µM of erlotinib (E100), or 100µM of the EGFR TKI plus escalating doses (10µM, 20µM, or 100µM) of the BCL6 inhibitor (BE10, BE20, BE100, respectively). The BCL6 inhibitor used, 'BCL6 inhibitor, 79-6' by Calbiochem, was a cell-permeable, selective inhibitor of the transcriptional repression activity of BCL6. We found that BCL6 mRNA expression was still elevated after EGFR TKI treatment even as we escalated its inhibition (Figure 14). This confirmed our suspicions that BCL6 upregulation is significant and sustained following EGFR TKI



treatment, an important indicator of potentially aberrant behavior.

Figure 14: BCL6 mRNA expression is upregulated despite direct pharmacologic inhibition when human lung cancer cells are treated with EGFR TKIs. We treated PC9 and HCC827 cells for 24 hours with either DMSO alone, 100μM of BCL6 inhibitor (B100), 100μM of erlotinib (E100), or 100μM of erlotinib plus 10, 20, or 100μM of BCL6 inhibitor (BE10, BE20, or BE100, respectively). In both PC9 and HCC827 cells, treatment with TKIs induces BCL6 upregulation even with increasing doses of BCL6 inhibitor. *$p<0.05$ vs. previous dose.

AICDA is downregulated upon pharmacologic inhibition of BCL6, countered with upregulation upon EGFR TKI treatment across human lung cancer cell lines.

The next important step in delineating whether AICDA expression is directly related to BCL6 upregulation was to treat lung cancer cell lines again for 24 hours with either DMSO alone, 100μM of BCL6 inhibitor (B100), 100μM of erlotinib or osimertinib (H1975 only) (E100 or O100), or 100μM of erlotinib or osimertinib plus 10, 20, or 100μM of BCL6 inhibitor (BE10/BO10, BE20/BO20, or BE100/BO100, respectively). Our results showed that BCL6 inhibition did indeed suppress AICDA expression, partly countering the upregulation resulting from EGFR TKI treatment (Figure 15). This would suggest that AICDA upregulation results from and is dependent upon BCL6 upregulation after EGFR TKI treatment.

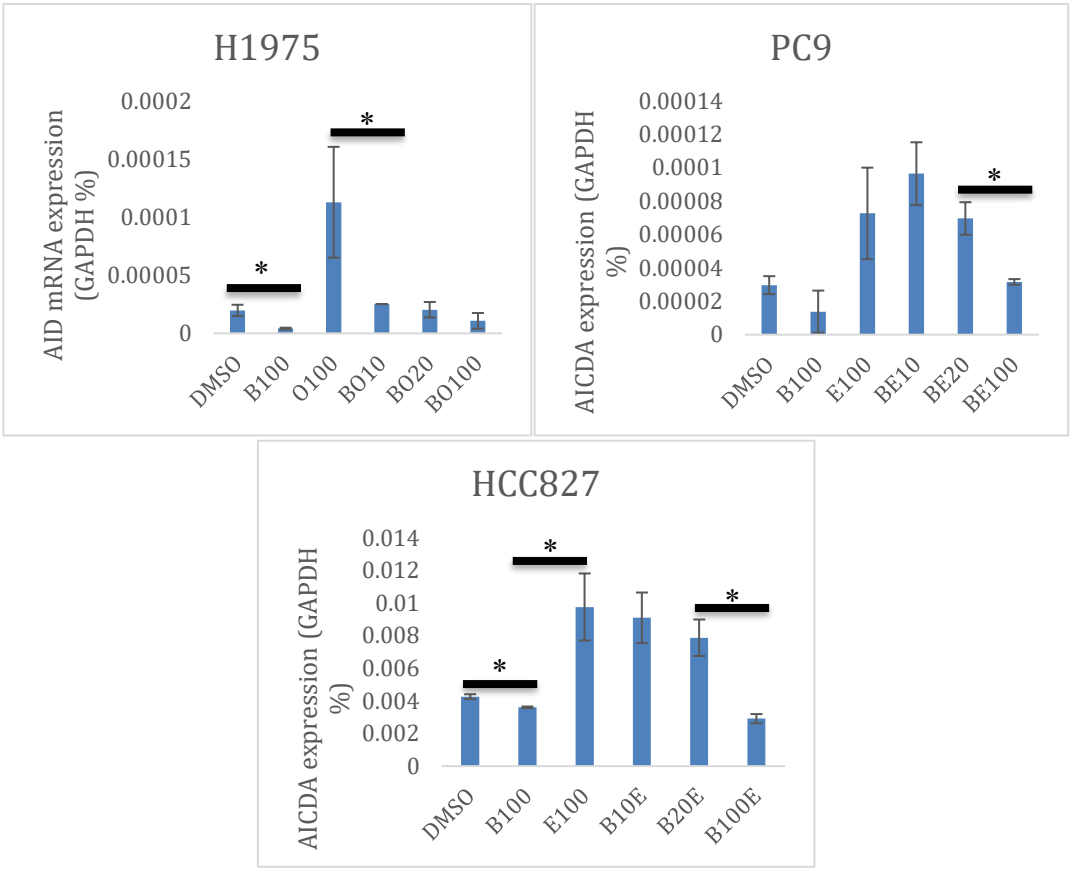


Figure 15: AID mRNA (AICDA) expression is decreased with BCL6 inhibition, countered by an upregulation in expression with EGFR TKI treatment. We treated H1975, PC9, and HCC827 cells for 24 hours with either DMSO alone, 100µM of BCL6 inhibitor (B100), 100µM of erlotinib or osimertinib (H1975 only) (E100 or O100), or 100µM of erlotinib plus 10, 20, or 100µM of BCL6 inhibitor (BE10/BO10, BE20/BO20, or BE100/BO100, respectively). With the exception of PC9 cells, all cell lines treated show a significant reduction in AICDA expression when treated with a BCL6 inhibitor alone, while EGFR TKI treatment increases expression enough to counter this decrease except at the highest dose of BCL6 inhibitor. *$p<0.05$ vs. preceding dose.

Patterns of APOBEC family mRNA expression upon treatment with both EGFR TKI and pharmacologic BCL6 inhibitor in human lung cancer cell lines.

Next, we sought to understand how APOBEC mRNA expression changes with BCL6 inhibition and EGFR TKI treatment combined. Again, we treated lung cancer cell lines for 24 hours with either DMSO alone, 100μM of BCL6 inhibitor (B100), 100μM of erlotinib or osimertinib (H1975 only) (E100 or O100), or 100μM of erlotinib or osimertinib plus 10, 20, or 100μM of BCL6 inhibitor (BE10/BO10, BE20/BO20, or BE100/BO100, respectively). Our results showed elevated and sustained expression with EGFR TKI treatment despite escalating BCL6 inhibition in APOBEC3A and APOBEC3B. In APOBEC3C, APOBEC3D, APOBEC3F, and APOBEC3G, we found similar results specifically in HCC827 cells but not PC9 cells (Figures 16-21). We believe this difference in behavior could be due to the cell lines' distinctive mutational profile. PC9 also carries mutant TP53 which could contribute to its variant response to EGFR TKI treatment (Meder et. al., 2016). That said, each APOBEC cytidine deaminase shows significant dependence on BCL6 expression.

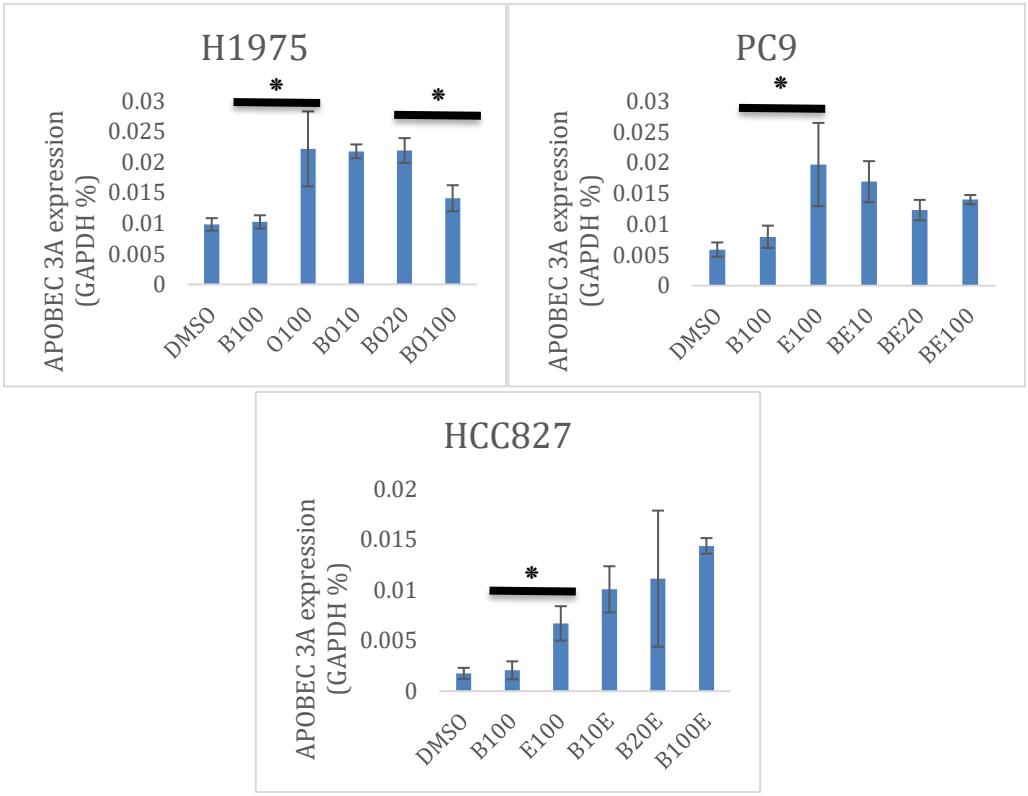


Figure 16: APOBEC3A is upregulated upon treatment with EGFR TKI regardless of BCL6 inhibition. We treated H1975, PC9, and HCC827 cells for 24 hours with either DMSO alone, 100μM of BCL6 inhibitor (B100), 100μM of erlotinib or osimertinib (H1975 only) (E100 or O100), or 100μM of erlotinib plus 10, 20, or 100μM of BCL6 inhibitor (BE10/BO10, BE20/BO20, or BE100/BO100, respectively). Of note, H1975 cells show downregulation of APOBEC3A expression when treated with osimertinib and the highest dose of BCL6 inhibitor, while PC9 and HCC827 exhibit no significant upregulation beyond initial erlotinib dose. *$p<0.05$ vs. preceding dose.

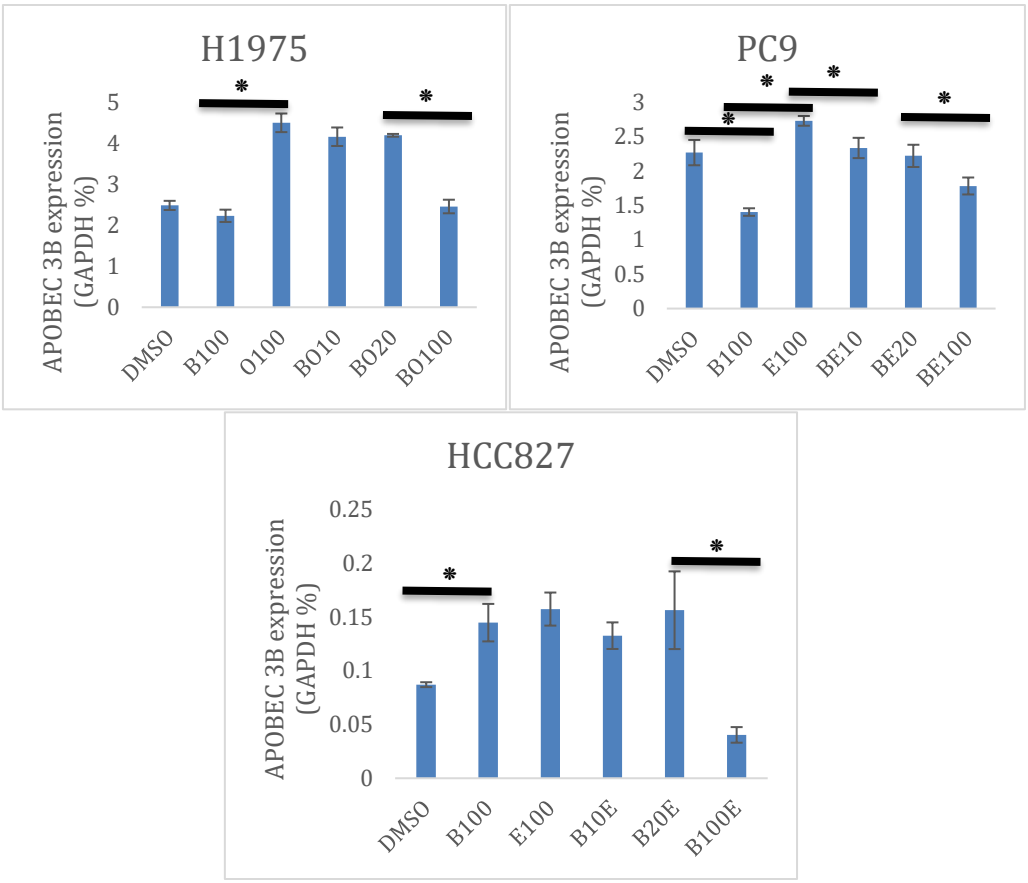


Figure 17: APOBEC3B mRNA expression is upregulated with EGFR TKI treatment and downregulated when treated with both EGFR TKI and the highest dose of BCL6 inhibitor. We treated H1975, PC9, and HCC827 cells for 24 hours with either DMSO alone, 100μM of BCL6 inhibitor (B100), 100μM of erlotinib or osimertinib (H1975 only) (E100 or O100), or 100μM of erlotinib plus 10, 20, or 100μM of BCL6 inhibitor (BE10/BO10, BE20/BO20, or BE100/BO100, respectively). Of note, APOBEC3B shows mixed response to initial treatment of BCL6 inhibitor alone among cell lines, with a significant upregulation in HCC827 cells, a significant downregulation in PC9 cells, and no significant change in H1975 cells. However, all cell lines show a significant downregulation when treated with EGFR TKI and the highest dose of BCL6 inhibitor. *$p<0.05$ vs. preceding dose.

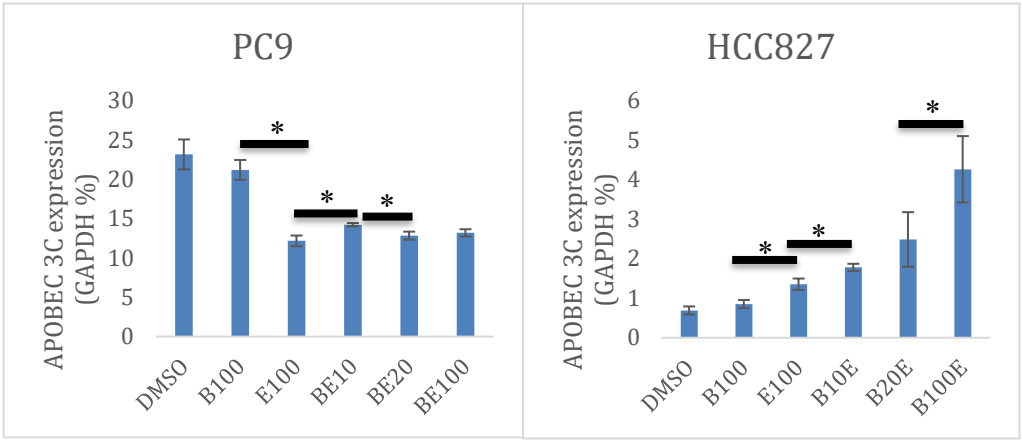
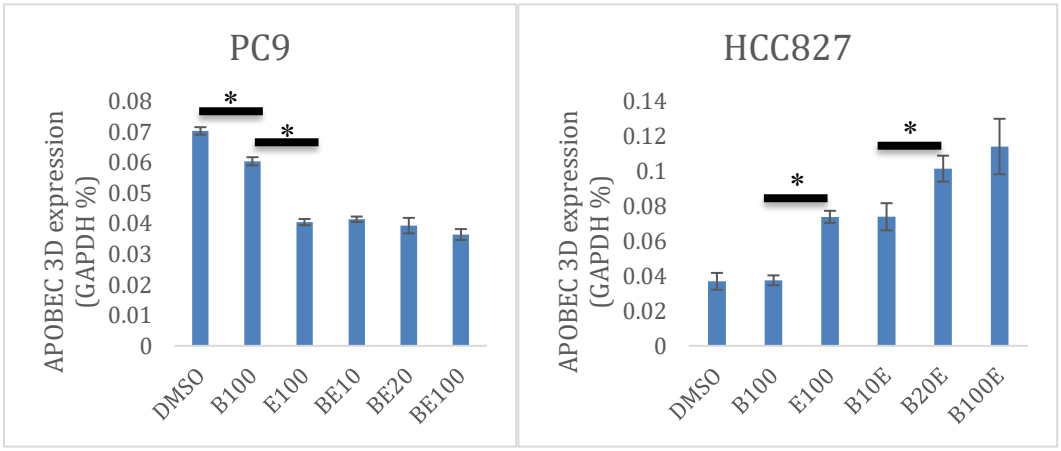


Figure 18: APOBEC3C mRNA expression is upregulated with EGFR TKI treatment despite BCL6 inhibition but varies by cell line. We treated PC9 and HCC827 cells for 24 hours with either DMSO alone, 100μM of BCL6 inhibitor (B100), 100μM of erlotinib (E100), or 100μM of erlotinib plus 10, 20, or 100μM of BCL6 inhibitor (BE10, BE20, or BE100, respectively). Notably, PC9 cells do not exhibit a similar



upregulation to HCC827 cells. *p<0.05 vs. preceding dose.

Figure 19: APOBEC3D mRNA expression is upregulated with EGFR TKI treatment despite BCL6 inhibition but varies by cell line. We treated PC9 and HCC827 cells for 24 hours with either DMSO alone, 100μM of BCL6 inhibitor (B100), 100μM of erlotinib (E100), or 100μM of erlotinib plus 10, 20, or 100μM of BCL6 inhibitor (BE10, BE20, or BE100, respectively). Of note, PC9 and HCC827 cells exhibit opposing behavior in response to BCL6 inhibition and EGFR TKI treatment, with PC9 cells exhibiting downregulation in both cases, and HCC827 cells exhibiting upregulation with TKI treatment and despite BCL6 inhibition. *p<0.05 vs. preceding dose.

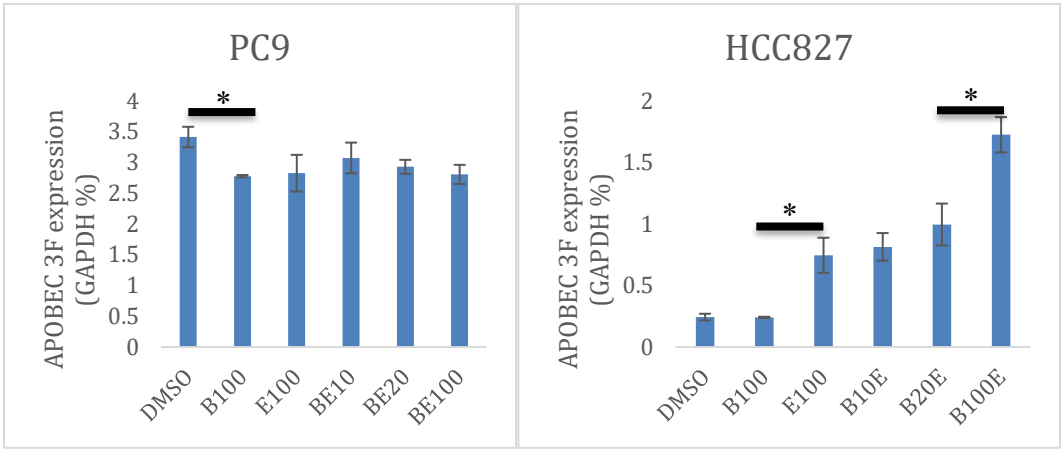


Figure 20: APOBEC3F mRNA expression is upregulated with EGFR TKI treatment despite BCL6 inhibition but varies by cell line. We treated PC9 and HCC827 cells for 24 hours with either DMSO alone, 100µM of BCL6 inhibitor (B100), 100µM of erlotinib (E100), or 100µM of erlotinib plus 10, 20, or 100µM of BCL6 inhibitor (BE10, BE20, or BE100, respectively). Once again, PC9 cells exhibit a downregulation in response to BCL6 inhibition while HCC827 cells exhibit an upregulation in response to EGFR TKI treatment despite BCL6 inhibition. *p<0.05 vs. preceding dose.

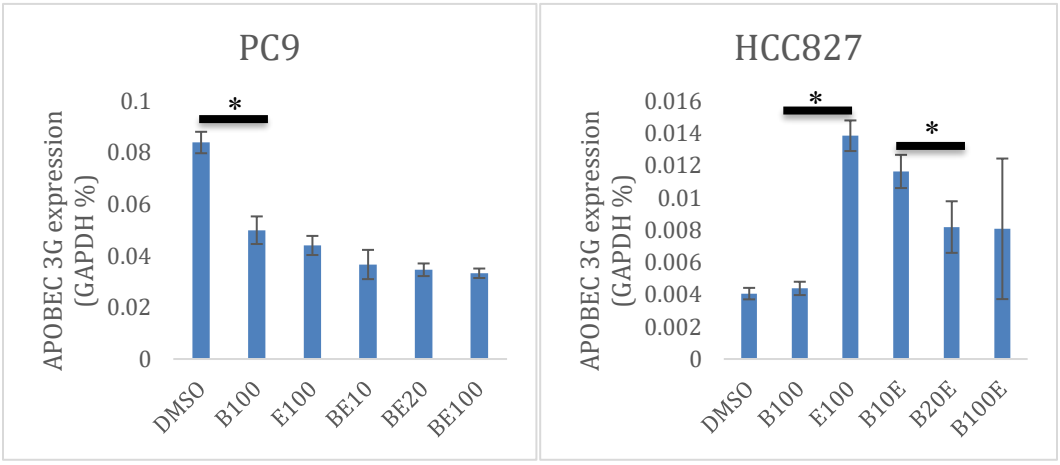


Figure 21: APOBEC3G mRNA expression is upregulated with EGFR TKI treatment but shows some downregulation when treatment is combined with BCL6 inhibition at escalating doses. We treated PC9 and HCC827 cells for 24 hours with either DMSO alone, 100µM of BCL6 inhibitor (B100), 100µM of erlotinib (E100), or 100µM of erlotinib plus

10, 20, or 100μM of BCL6 inhibitor (BE10, BE20, or BE100, respectively). Interestingly, once again, PC9 cells show an immediate downregulation upon inhibition of BCL6 with no upregulation in response to EGFR TKI treatment, while HCC827 cells show an increase in expression with EGFR TKI treatment. However, in HCC827 cells, APOBEC3G expression is also downregulated in response to escalating doses of BCL6 inhibition in combination with EGFR TKI treatment. *$p<0.05$ vs. preceding dose.

Patterns of AID and APOBEC mRNA expression upon transient over-expression of BCL6.

The next and final step in this series of experiments was to reveal the influence BCL6 has on expression of AICDA and the APOBEC series. We sought to find out how, when BCL6 is overexpressed in these cell lines, the expression of these downstream components of our hypothesized pathway respond (Figure 25). To do so, we introduced BCL6 DNA expression to human lung cancer cell lines at increasing levels via transient DNA transfection for 24 hours, using an empty vector for a control group. Again, with varying results by cell line, APOBEC3A, APOBEC3B, APOBEC3F, and APOBEC3G showed the most notable and consistent increases in expression following BCL6 overexpression, although APOBEC3C and APOBEC3D also had elevated expression (Figures 22-24). A consistently significant result of increased AICDA expression following BCL6 overexpression was not found in this experiment, although in HCC827 cells, AICDA expression was elevated at the lowest dose. We attribute this seemingly conflicting result to

extremely low endogenous expression, making it difficult to capture expression levels. However, our findings lead us to believe that there may be one or more alternate pathways aside from BCL6 that directly affect AICDA expression, dampening its response to BCL6 overexpression. This would reasonably explain both its dependence upon BCL6 and its small or lack of response to BCL6 overexpression.

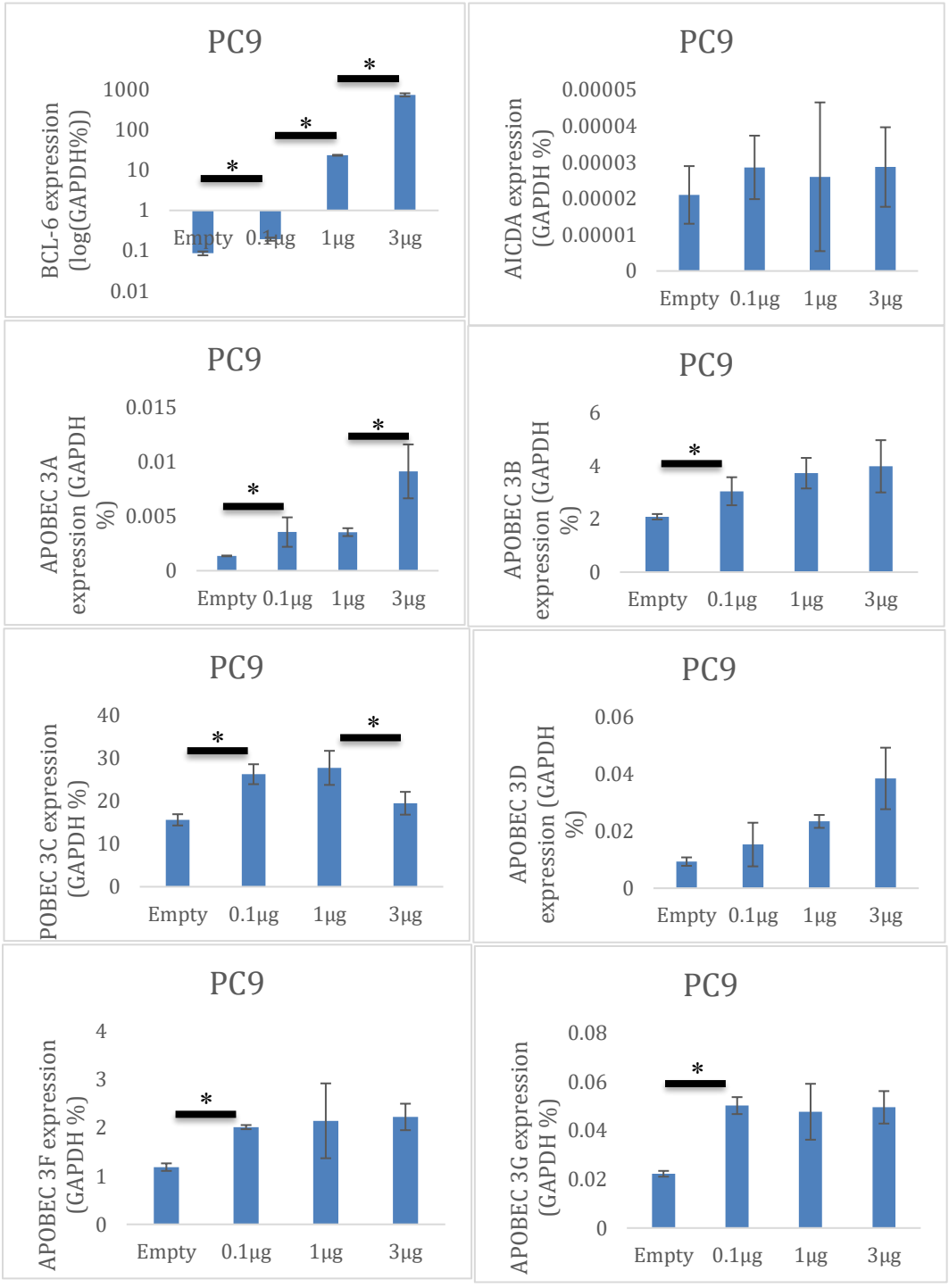
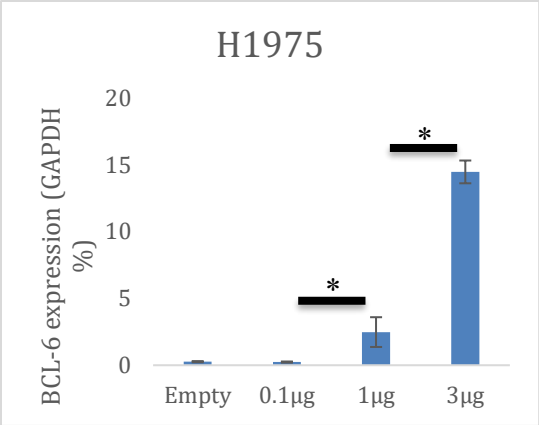
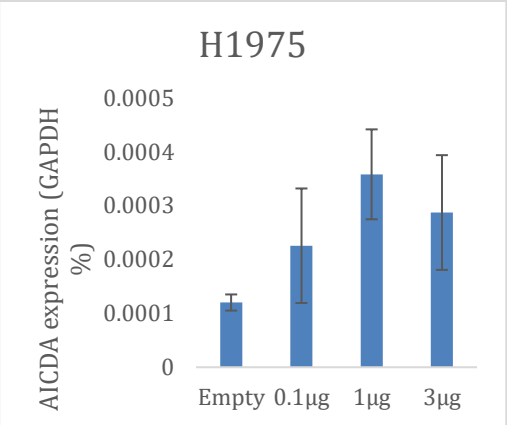
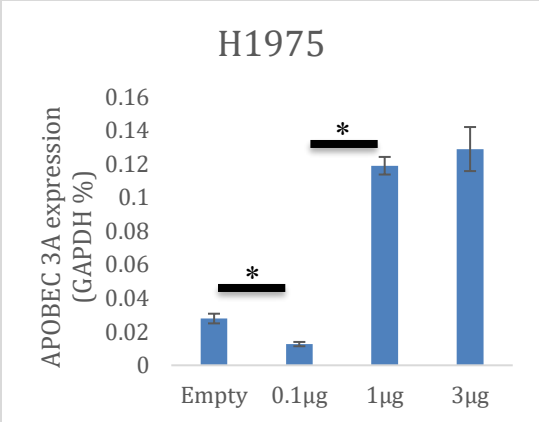


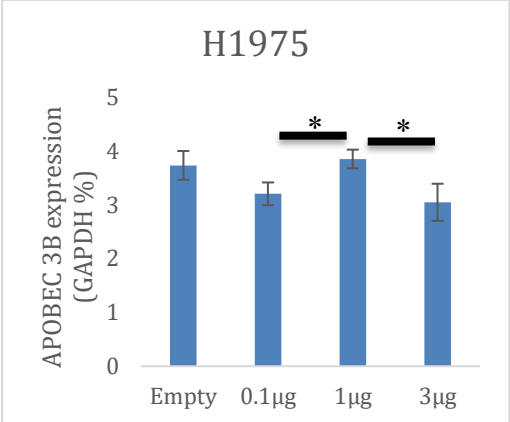
Figure 22: BCL6, AID, and APOBEC mRNA expression in PC9 cells treated with transient BCL6 overexpression. We treated PC9 cells with increasing doses of BCL6 DNA via transient transfection for 24 hours, using an empty vector for a control group. AID mRNA (AICDA) expression shows no significant response to BCL6 overexpression, while

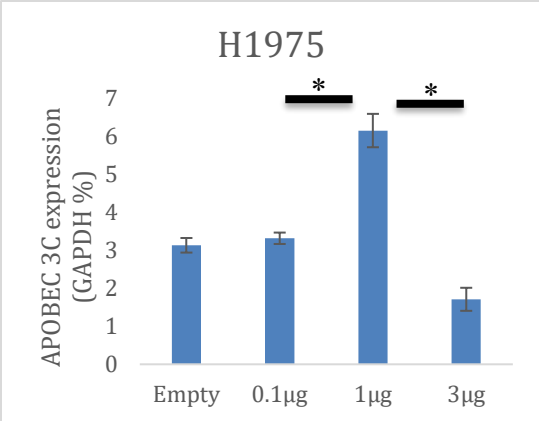
APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3F, and APOBEC3G are upregulated upon at 0.1µg. APOBEC3A is again upregulated at 3µg. Of note, BCL6 mRNA expression was dramatically increased at increasing doses of the DNA transfection, so we represented it as a log scale for improved visual representation. *$p<0.05$ vs. preceding dose.

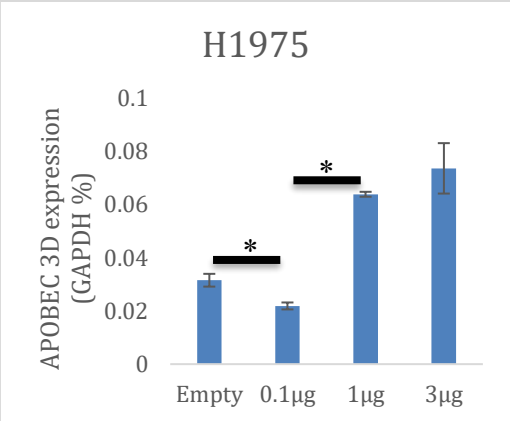

H1975
BCL-6 expression (GAPDH %)
20
15
10
5
0
*
*
Empty
0.1µg
1µg
3µg

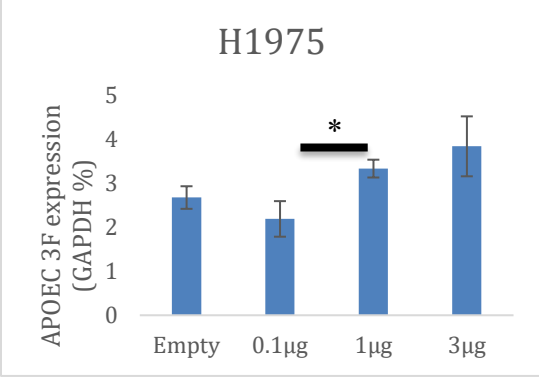

H1975
AICDA expression (GAPDH %)
0.0005
0.0004
0.0003
0.0002
0.0001
0
Empty
0.1µg
1µg
3µg


H1975
APOBEC 3A expression (GAPDH %)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
*
Empty
0.1µg
1µg
3µg


H1975
APOBEC 3B expression (GAPDH %)
5
4
3
2
1
0
*
*
Empty
0.1µg
1µg
3µg


H1975
APOBEC 3C expression (GAPDH %)
7
6
5
4
3
2
1
0
*
*
Empty
0.1µg
1µg
3µg


H1975
APOBEC 3D expression (GAPDH %)
0.1
0.08
0.06
0.04
0.02
0
*
*
Empty
0.1µg
1µg
3µg


H1975
APOEC 3F expression (GAPDH %)
5
4
3
2
1
0
*
Empty
0.1µg
1µg
3µg

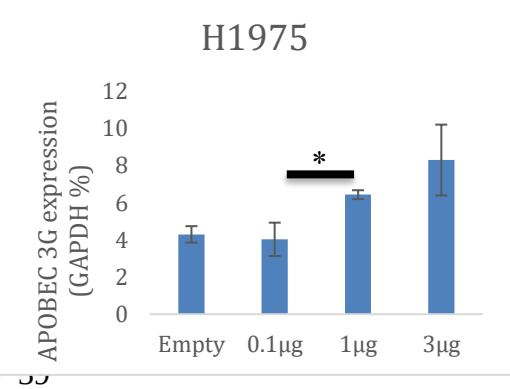

H1975
APOBEC 3G expression (GAPDH %)
12
10
8
6
4
2
0
*
Empty
0.1µg
1µg
3µg

Figure 23: BCL6, AID, and APOBEC mRNA expression in H1975 cells treated with transient BCL6 overexpression. We treated H1975 cells with increasing doses of BCL6 DNA via transient transfection for 24 hours, using an empty vector for a control group. AID mRNA (AICDA) expression showed no significant changes with overexpression, while APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, and APOBEC3G were all upregulated at 1 µg. Of note, APOBEC3B and APOBEC3C also showed significant downregulation at 3µg. *$p<0.05$ vs. preceding dose.

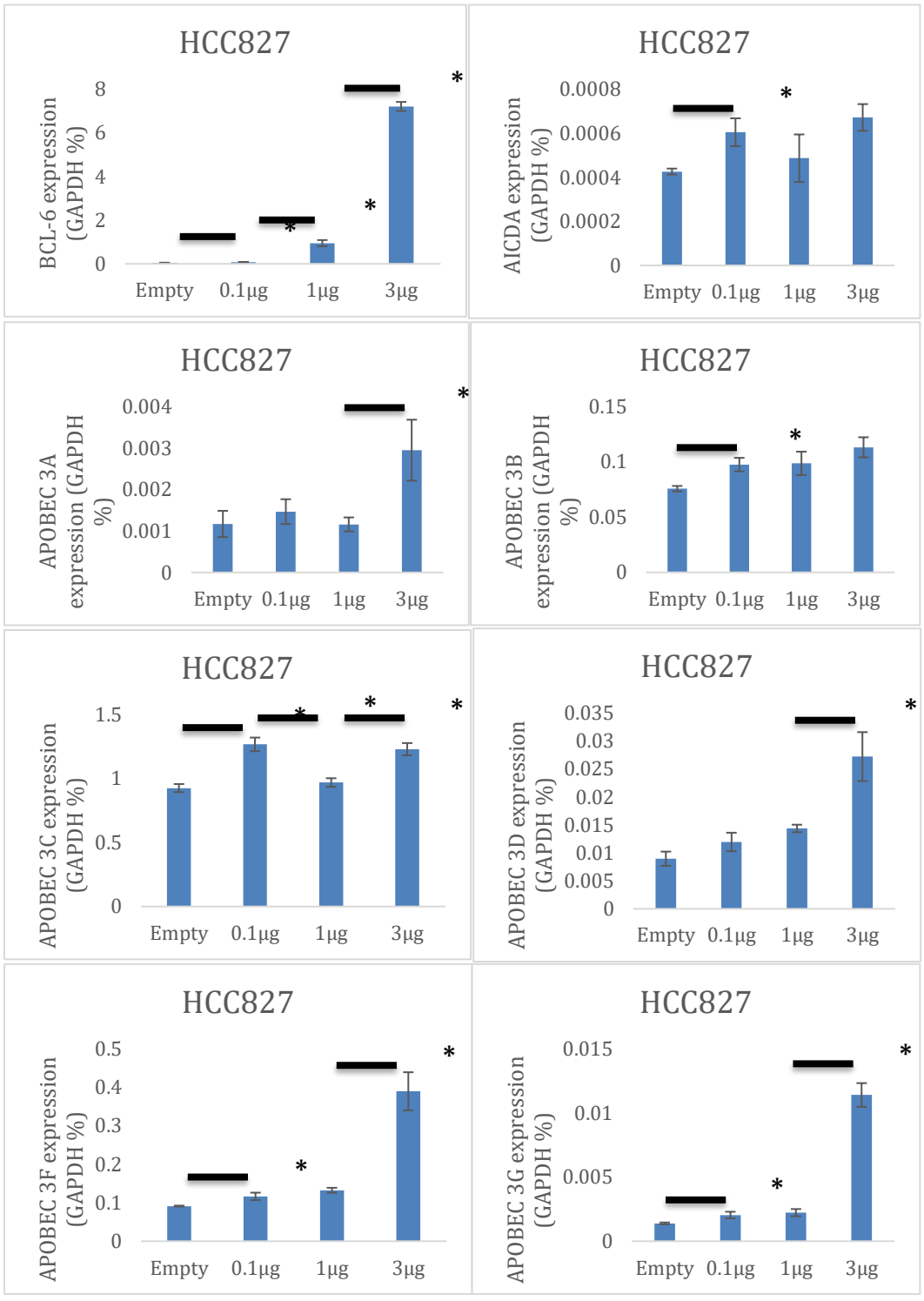


Figure 24: BCL6, AID, and APOBEC mRNA expression in HCC827 cells treated with transient BCL6 overexpression. We treated HCC827 cells with increasing doses of BCL6 DNA via transient transfection for 24

hours, using an empty vector for a control group. AID mRNA (AICDA) expression was upregulated at 0.1µg, as was APOBEC3B and APOBEC3C, although APOBEC3C showed fluctuating expression at higher doses. APOBEC3A and APOBEC3D were both upregulated at 3µg, and APOBEC3F and APOBEC3G were both upregulated at both 0.1 and 3µg. *$p<0.05$ vs. preceding dose.

DISCUSSION

I sought to shed light on the pathway behind the development of the T790M mutation that confers resistance to EGFR TKIs in mutant-EGFR NSCLC, building on work that had begun in the Kobayashi lab. The Kobayashi lab began by studying the potential role AID plays in this process because of its normal function of deaminating cytosine to produce uracil, resulting in C/G to T(U)/A transitions in DNA and/or RNA. Those results show that erlotinib directly induces a significant increase in AID mRNA expression (referred to by the gene name AICDA), in mutant-EGFR cell lines but not in cell lines with wild-type EGFR. Furthermore, when PC9 cells are erlotinib-resistant, AICDA expression is increased more, and the erlotinib resistance is due to the development of T790M. Next, by stably expressing AID in BaF3 cells which do not normally express it, they showed that AID is sufficient to give rise to the T790M mutation in EGFR and resistance to erlotinib. Thus, AID is at least partially responsible for the T70M mutation in this setting. When Gilbert Pan and I treated several mutant-EGFR lung cancer cell lines with EGFR TKIs, we found that AICDA expression did not necessarily rise in a dose-dependent fashion, which was a surprising result with unclear significance

at this point. This result may point to a more complex relationship between EGFR TKIs and AID.

Next, I investigated the potential role BCL6 might play in this mechanism because of its regulatory role over AID in the normal immune response. Upon treatment with EGFR TKIs, mutant-EGFR lung cancer cell lines showed an increase in BCL6 mRNA expression. In contrast to no significant increase in BCL6 mRNA expression observed in normal bronchial cells. This indicates that BCL6 activity may increase after EGFR TKI treatment. Next, we treated these lung cancer cells with combinations of EGFR TKI and a pharmacological BCL6 inhibitor, 'BCL6 inhibitor, 79-6' by Calbiochem, which is a cell-permeable, selective inhibitor of the transcriptional repression activity of BCL6 and thus induces expression of BCL6 target genes. It works by binding the critical site within the corepressor binding domain of BCL6, thus disrupting BCL6/corepressor complexes (Cerchietti et. al., 2010). We found the increase in BCL6 mRNA expression was sustained despite this direct inhibition. This indicates that treatment with a TKI is very effective in maintaining high levels of BCL6. We found that AICDA expression was reduced with BCL6 inhibition, and escalating inhibition dampened the usual increase in AICDA expression upon EGFR TKI treatment. This shows that AICDA upregulation may be dependent upon BCL6 mRNA upregulation. Interestingly, upon transient BCL6 overexpression, we did

not observe an increase in AICDA expression. This could be due to its very low endogenous expression. However, we surmise that AID could be a common point in merging pathways that causes a dampened response to BCL6 overexpression. Specifically, Kadi *et. al.* found that AICDA expression is induced by the NFκB pathway (Kadi et. al. 2018). We would suggest that this is one possible component of a dual pathway that induces AICDA expression upon EGFR TKI treatment.

The final piece of our investigation is of the family of cytidine deaminases that we suggest may also play a role in the development of the C to T transition responsible for T790M, as their normal function in the immune response is to deaminate cytosine to produce uracil, resulting in C/G to U/A transitions in mRNA. We found that increases in expression upon EGFR TKI treatment were most prevalent in APOBEC3B, APOBEC3D, but present in others across most cell lines. This would indicate that members of the APOBEC family may be more active in mRNA editing, leading to the C to T transition, after EGFR TKI treatment. We also found that BCL6 inhibition did not necessary impede APOBEC expression, which could indicate an indirect relationship following increases in BCL6 expression. However, we did observe an increase in expression across all APOBECs when BCL6 was overexpressed. More study is required to delineate the role of these

cytidine deaminases in this mechanism, but our results suggest a strong potential for involvement, most likely following BCL6.

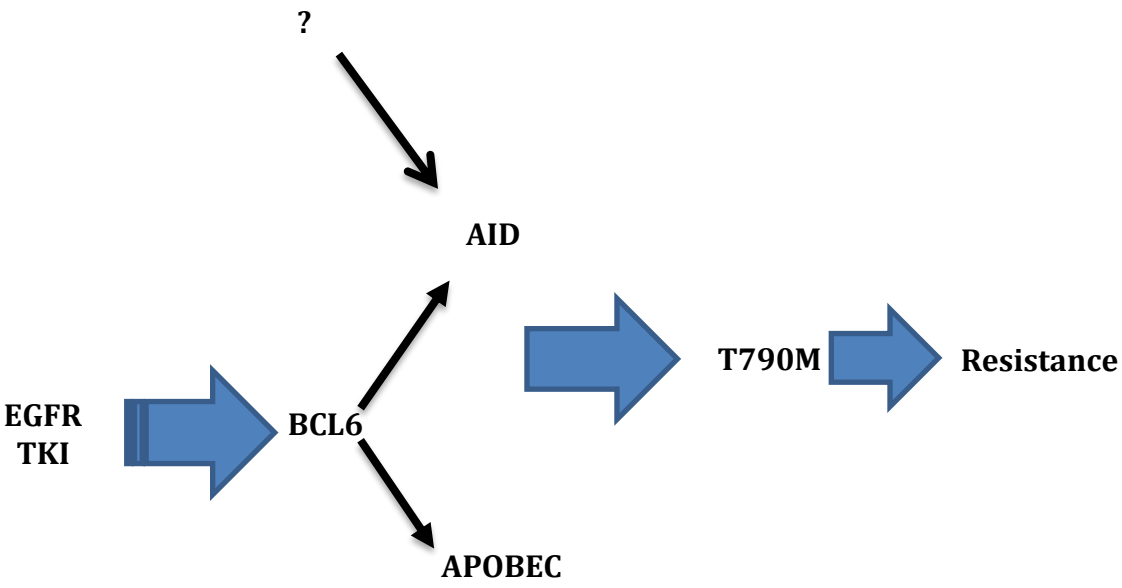


Figure 25: Suggested pathway leading to T790M which confers resistance to EGFR TKIs in mutant-EGFR NSCLC. Our results support the pathway shown above. In mutant-EGFR NSCLC, EGFR TKI treatment leads to upregulation of BCL6 mRNA, which in turn leads to upregulation of AICDA and mRNA of members of the APOBEC family of cytidine deaminases. AID and APOBECs deaminate cytosine to produce uracil, which leads to T790M. T790M is a common mutation that confers resistance to EGFR TKIs in this setting.

In conclusion, this thesis suggests that in mutant-EGFR NSCLC, treatment with EGFR TKI induces both BCL6 mRNA expression and AID mRNA expression, as well as increased mRNA expression in varying APOBECs, perhaps most notably APOBEC3B and APOBEC3D, and that this is the mechanism by which the C to T transition arises, leading to T790M which confers resistance to EGFR TKIs (Figure 25). We also suggest that AID is sufficient to produce T790M, and that there may be an

alternative mechanism driving AICDA expression and subsequent behavior. Further studies to build on our findings would include analyzing AID, BCL6, and APOBEC expression changes under the same conditions as discussed here at the protein level using Western blotting. Taken together, it could be worthwhile to pursue these targets *in vivo* once they are better defined.

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